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A Fifteen-Amino-Acid Peptide Inhibits Human Papillomavirus E1-E2 Interaction and Human Papillomavirus DNA Replication In Vitro

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Mutation of the conserved glutamic acid residue at position 39 of human papillomavirus type 16 (HPV-16) E2 to alanine (E39A) disrupts its E1 interaction activity and its replication function in transient replication assays but does not affect E2 transcriptional activation. This E39A mutation also disrupts replication activity of HPV-16 E2 in HPV-16 in vitro DNA replication. On this basis, we designed 23- and 15-amino-acid peptides derived from HPV-16 E2 sequences flanking the E39 residue and tested the ability of these peptides to inhibit interaction between HPV-16 E1 and E2 in vitro. The inhibitory activity of these peptides was specific, since analogous peptides in which alanine was substituted for the E39 residue did not inhibit interaction. The 15-amino-acid peptide E2N-WP15 was the smallest peptide tested that effectively inhibited HPV-16 E1-E2 interaction. This peptide also inhibited in vitro replication of HPV-16 DNA. The efficacy of E2N-WP15 was not exclusive to HPV-16: this peptide also inhibited interaction of HPV-11 E1 with the E2 proteins of both HPV-11 and HPV-16 and inhibited in vitro replication with these same combinations of E1 and E2 proteins. These results provide further evidence that E1-E2 interaction is required for papillomavirus DNA replication and constitute the first demonstration that inhibition of this interaction is sufficient to prevent HPV DNA replication in vitro.

The papillomaviruses are small, circular double-stranded DNA viruses that cause epithelial lesions. Of the greater than 70 known human papillomaviruses (HPVs), those associated with anogenital lesions have been classified into two general categories, based on the relative tendencies of their associated lesions to remain dysplastic but benign (termed low-risk HPVs) or to progress to high-grade intraepithelial neoplasias and to invasive malignant tumors (high-risk HPVs). At least 90% of human cervical cancers contain viral sequences of a high-risk HPV subtype (i.e., HPV-16, HPV-18, HPV-31, or HPV-33). Of the high-risk HPVs, HPV-16 is the predominant subtype and is found in 50 to 70% of human cervical cancers (48). Our laboratory has focused on HPV-16 as a particularly important target for the study of functions associated with papillomavirus oncogenesis and viral pathogenesis.

The papillomaviruses, like simian virus 40 (SV40), have served as a model system for the study of mammalian DNA replication. In vitro replication studies have shown that papillomavirus and SV40 resemble each other in their utilization of a number of cellular replication proteins, including DNA polymerase α primase (4, 30), DNA polymerase δ, RPA, PCNA, and topoisomerases (18, 26, 29, 43), although papillomavirus DNA replication may also require some additional cellular factors (26). Two virus-encoded proteins, E1 and E2, are required for the initiation of papillomavirus DNA replication (40). The papillomavirus E1 protein is the replication enzyme that initiates viral DNA replication. The E1 proteins encoded by the various papillomaviruses are well conserved and bear significant homology to the large T antigen replication protein of SV40 (8, 23). Both the papillomavirus E1 proteins and SV40 large T antigen bind to a region of dyad symmetry within their respective replication origins (14, 15, 27, 41) and possess ATP-dependent DNA helicase activities (16, 17, 36, 37, 44). In comparison to T-antigen-dependent SV40 replication, however, very little is known about the biochemical events and cellular activities involved in papillomavirus replication.

Unlike SV40 large T antigen, E1 does not bind the viral replication origin autonomously. Instead, the papillomavirus E2 protein is required as a cofactor for E1 binding to the origin (3, 28, 32). The papillomavirus E2 proteins are composed of two functional domains: an amino-terminal transcriptional activation domain and a carboxy-terminal DNA binding domain that binds as a dimer to the $ACCN_6GGT$ recognition sequence (2, 24, 25). These domains of E2 are separated by a less-conserved, dispensable hinge region. The amino-terminal domain of E2 serves two functions in the viral life cycle, both as a transcriptional regulator of viral gene expression and as an E1 interaction domain that is required for recruitment of the E1 replication protein to the papillomavirus origin of replication. E2 binding sites flank the replication origin. By binding to these sites and through interaction with E1, E2 directs E1 to the papillomavirus replication origin (11, 21, 22, 34–36, 39, 43). This interaction is thought to facilitate recognition of the viral replication origin by E1 and subsequent assembly of the replication initiation complex, including recruitment of the DNA polymerase α priming enzyme (4, 30), and is essential for efficient viral DNA replication in transient transfection assays (31, 32).

The results presented here show that we have achieved replication of HPV-16 DNA in vitro. This replication mimics that seen in transient in vivo replication assays in that it requires interaction of E2 with E1 (31, 32). Furthermore, we demonstrate the ability of a 23- or 15-amino-acid peptide derived from the HPV-16 E2 amino terminus to disrupt both interaction between E1 and E2 and papillomavirus DNA replication in vitro. The activity of such a peptide as a functional inhibitor

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in these assays has several implications: (i) it identifies a discrete region of E2 that is necessary and sufficient for specific interaction with E1, (ii) it provides proof of the concept that a relatively small molecule may be used to inhibit E1-E2 interaction and papillomavirus DNA replication, (iii) it provides a starting point for the design or discovery of biologically active molecules capable of inhibiting papillomavirus DNA replication, and (iv) it provides a reagent that may facilitate functional dissection of the dynamic processes that result in the stepwise assembly of the papillomavirus DNA replication initiation complex. These observations may aid in the identification of effective bioactive antiviral compounds that would inhibit HPV DNA replication.

MATERIALS AND METHODS

Baculovirus expression of HPV E1 and E2 proteins. An *Nco*I-*Sma*I fragment from pUC-$E1_{16}$ (9) containing the HPV-16 E1 protein coding region was made blunt by using T4 polymerase and inserted into the blunt-ended *Bam*HI site of pBS-EE to generate pBS-EE-$E1_{16}$. [pBS-EE was generated by cloning the *Xba*I-*Bam*HI fragment encoding the polyomavirus middle T antigen EE epitope (18) from pUC-EE into pBluescript-SK(+) (Stratagene).] The *Eco*RI-*Not*I fragment from pBS-EE-$E1_{16}$ was recloned into the pVL1392 baculovirus transfer vector (Pharmingen). HPV-16 E2 and HPV-16 E2 E39A were cloned as *Bam*HI-*Eco*RI fragments into pVL1392.

Recombinant baculovirus expressing HPV-11 EE-E1 and HPV-11 E2 were generously provided by Louise Chow. Hi-Five cells were infected at a multiplicity of 5 to 10 PFU per cell and incubated at 27°C for 48 h. Cells were scraped into ice-cold phosphate-buffered saline, pelleted by centrifugation in a Sorvall RT6000D tabletop centrifuge at 1,000 rpm for 5 min, washed once in ice-cold phosphate-buffered saline, and repelleted. The cell pellet was then resuspended in hypotonic buffer (buffer A) containing 10 mM HEPES-K^+ (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), and protease inhibitor cocktail (PIC; 1 mM phenylmethylsulfonyl fluoride, 10-μ-g/ml aprotinin, 10-μg/ml leupeptin, 10-μg/ml pepstatin A, 10-μg/ml phenanthroline, 16-μg/ml benzamidine). Cells were pelleted as before, resuspended in buffer A containing 0.5% Nonidet P-40 (NP-40), and incubated on ice for 10 min. Lysates were then spun at 4°C in a Sorvall tabletop microcentrifuge (3,000 rpm). The cytoplasmic extract supernatant was removed, and an equal volume of 50% (vol/vol) glycerol was added. Lysates were quick-frozen on dry ice and stored at −70°C until use. The nuclear pellet was washed once in buffer A without NP-40, resuspended in buffer C (20 mM HEPES-K^+ [pH 7.9], 429 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% [vol/vol] glycerol, 0.5 mM DTT containing PIC), and incubated on ice for 30 to 40 min with intermittent vortexing. After clarification by centrifugation at full speed in a Sorvall microcentrifuge for 10 min at 4°C, the nuclear extract was quick-frozen on dry ice and stored at −70°C until use.

In vitro HPV E1-E2 binding assay. Protein (300 to 500 ng) from HPV-16 or HPV-11 EE-E1-expressing recombinant baculovirus-infected cell nuclear extracts and 80 to 120 ng from HPV E2-expressing baculovirus-infected cell nuclear extracts were incubated in 500 μl of NET-gel buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 0.25% gelatin) at 4°C for 1 h. Anti-EE monoclonal antibody (1 to 2 μg) was added, and the mixture was incubated with shaking at 4°C for 1 h. Immune complexes were precipitated with protein G-agarose beads and washed twice for 30 min with ice-cold NET-gel buffer. Precipitated proteins were detected by Western blotting with appropriate primary antibodies and anti-rabbit immunoglobulin G-horseradish peroxidase or anti-mouse immunoglobulin G-horseradish peroxidase (Amersham). Membranes were blocked for 1 h in TNET (10 mM Tris [pH 7.5], 2.5 mM EDTA, 50 mM NaCl, 0.1% Tween 20) containing 4% (wt/vol) dry milk. Washes and antibody reactions were carried out in TNET, and proteins were visualized by chemiluminescence (Renaissance ECL; NEN) and autoradiography. Bands were quantitated by using the NIH image program.

Purification of HPV EE-E1 and E2 proteins. EE-E1-containing, baculovirus-infected cell nuclear extract (3 to 5 mg of total protein) containing 1% NP-40 was passed over a mono-Q column (Econo-Pac Q cartridge, Bio-Rad no. 732-0025). The flowthrough was incubated at 4°C for 1 h with 200 μl of anti-EE monoclonal antibody that had been covalently cross-linked to protein G-Sepharose beads. Beads were pelleted by centrifugation and washed once with 800 μl of (1%) NP-40 lysis buffer with PIC. Beads were then washed three times for 10 min each time at 4°C with 800 μl of high-salt buffer (20 mM Tris [pH 7.0], 0.5 M NaCl, 0.5 mM DTT, 0.1% NP-40, PIC), followed by three 10-min washes with 800 μl of sodium phosphate buffer (10 mM $NaPO_4$ [pH 8.0], 0.5 mM DTT, 0.1% NP-40, PIC). EE-E1 was eluted from the pelleted beads in 100 mM triethylamine (pH 11.5). The beads were pelleted by centrifugation at 4°C, and the supernatant was dialyzed for 3 h at 4°C against 1 liter of dialysis buffer (HEPES-K^+ [pH 7.5], 1 mM DTT, 10% glycerol, 150 mM NaCl). The dialysis buffer was then changed, and dialysis was continued overnight at 4°C. This procedure yielded approximately 100 to 150 ng of EE-E1 (>95% pure) per μl. Twenty-microliter aliquots of purified EE-E1 were quick-frozen on dry ice and stored at −70°C until use.

Baculovirus-infected cell nuclear extract containing E2 in buffer C (3 mg/ml) was applied to an Econo-Pac heparin column cartridge (Bio-Rad no. 732-0075) at 4°C. After washing with 5 column volumes of buffer C containing PIC at a flow rate of 1 ml/min, E2 was eluted in 5 column volumes of buffer C containing 0.7 M NaCl and 0.5-ml column fractions were collected. Fractions containing E2 were determined by Coomassie staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels loaded with aliquots of eluted fractions. This procedure yielded 300 to 500 ng of E2 per μl, which was stored at −70°C until use.

In vitro replication reactions. In vitro replication reactions were prepared essentially as described by Kuo et al. (18), in 25-μl reaction mixtures at 37°C for different times. Standard reactions lasted 2 h and were terminated by addition of 200 μl of stop solution (20 mM Tris-HCl [pH 7.5], 10 mM EDTA, 0.1% sodium dodecyl sulfate, 20-μg/ml RNase A). After incubation at 37°C for 15 min, proteinase K was added (200 μg/ml) and incubation was continued for 30 min. Samples were extracted with 250 μl of phenol-chloroform-isoamyl alcohol (25:24:1), ethanol precipitated, washed with 70% ethanol, dried, resuspended in 20 to 30 μl of TE (10 mM Tris [pH 8.0], 1 mM EDTA), and analyzed on a 0.8% agarose gel. The agarose gel was dried onto a Hybond N^+ membrane (Amersham), and replicated DNA was visualized by autoradiography. Bands were quantitated by PhosphorImager analysis. Replication templates were pKS7838-7905 (nucleotides 7838 to 7905 and 1 to 130) containing the HPV-16 replication origin and pUC7874-99 containing the HPV-11 origin of replication (nucleotides 7874 to 7933 and 1 to 99). pUC7874-99 was generously provided by Louise Chow.

RESULTS

In vitro replication of HPV-16 DNA. Transient replication assays have demonstrated that interaction between HPV-16 E1 and E2 is required for efficient origin-dependent papillomavirus replication in vivo (31). E2 stimulation of bovine papillomavirus (BPV) and HPV-11 replication has been previously demonstrated in vivo (9, 19, 38, 40) and in vitro (18, 19, 43). We sought to establish a system for the study of HPV-16 DNA replication in vitro and to determine whether specific interaction between HPV-16 E1 and E2 is required for HPV-16 DNA replication in this system. Fig. 1A shows replication of an HPV-16 origin-containing plasmid in vitro using 293 cell extracts, along with partially purified HPV-16 E2 and increasing amounts of affinity-purified HPV-16 EE-E1. As has been documented for other papillomavirus E1 proteins, some replication was observed when relatively large amounts of E1 were used in the absence of E2 (lanes 2 through 7) (5, 6, 16, 22, 33, 40, 48). Addition of 6 ng of E2 greatly stimulated in vitro replication (lanes 8 to 13), whereas no replication was observed in the absence of EE-E1 (lane 1). Replication in this assay is origin dependent, since the small amount of replication of the pKS plasmid lacking the HPV-16 origin detected at high concentrations of EE-E1 (Fig. 1B, lanes 1 to 6) was not stimulated by E2 (Fig. 1B, lanes 7 to 12). Quantitation of these results is shown in Fig. 1C.

E1 interaction is required for E2 stimulation of HPV-16 replication in vitro. We have previously demonstrated that substitution of alanine for the conserved glutamic acid residue at amino acid residue 39 of HPV-16 E2 (E39A) disrupts both HPV-16 E1 interaction and transient HPV-16 DNA replication in transient cotransfection assays. E39A is, however, fully competent as a transcriptional activator (31). To test the capacity of this mutant E2 protein to support HPV-16 DNA replication in vitro, increasing amounts of wild-type HPV-16 E2 or the E39A HPV-16 E2 mutant protein were tested for in vitro replication activity in the presence of 20 ng of EE-E1 (Fig. 2A). Wild-type E2 greatly stimulated in vitro replication in a dose-responsive manner, whereas the E39A mutant of HPV-16 E2, which does not interact with E1, had only a slight stimulatory effect, even at relatively high concentrations. This result demonstrated the dependence of E2 amino-terminally mediated interaction with E1 in in vitro replication. Quantitation of these results is shown in Fig. 2B.

Inhibition of interaction between HPV-16 E1 and E2 in vitro. The results described above suggested that a region of

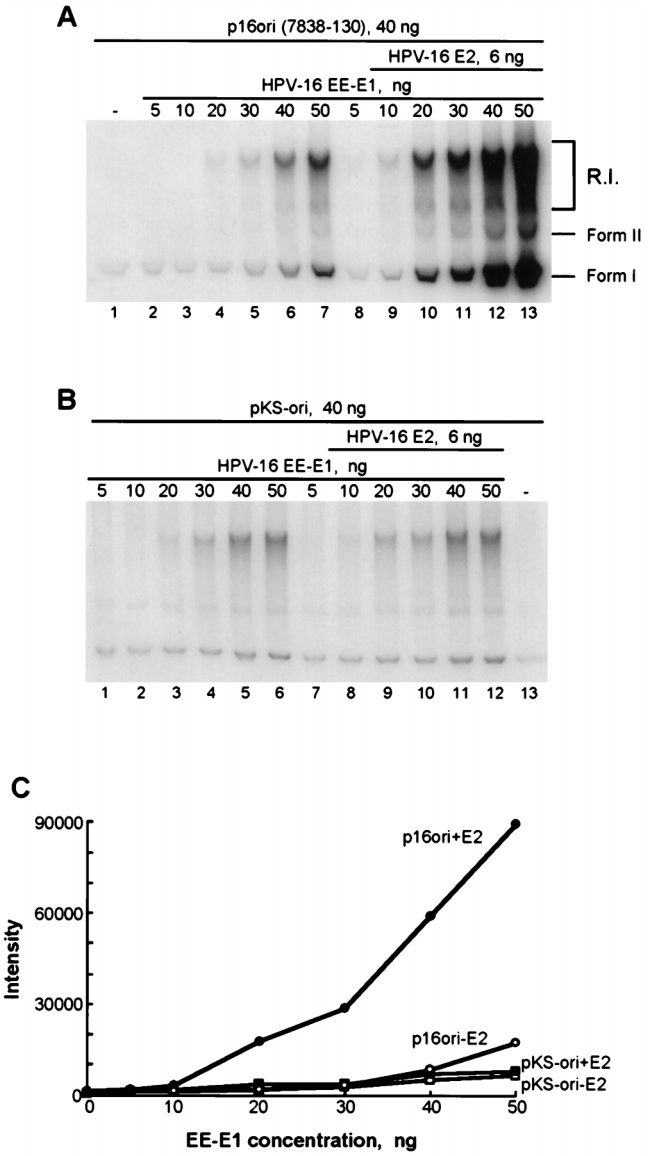

FIG. 1. E2 stimulates replication of HPV-16 in vitro. (A) Replication of HPV-16 origin-containing plasmid p16ori, a Bluescript KS+ plasmid containing HPV-16 genomic sequences (7838-139), by using 293 cell extract and increasing amounts of EE-16E1 (10 to 50 ng) in the presence (lanes 8 to 13) or absence (lanes 2 to 7) of HPV-16 E2. The reaction mixture in lane 1 contained no HPV-16 E1 or E2. The positions of replicative intermediates (R.I.) and form I and II DNAs analogous to those observed by Kuo et al. (18) are indicated. (B) In vitro HPV-16 DNA replication is origin dependent. Replication of Bluescript KS+ that does not contain HPV-16 origin sequences under conditions identical to those described for panel A is shown. (C) PhosphorImager quantitation of the replication experiments shown in panels A and B.

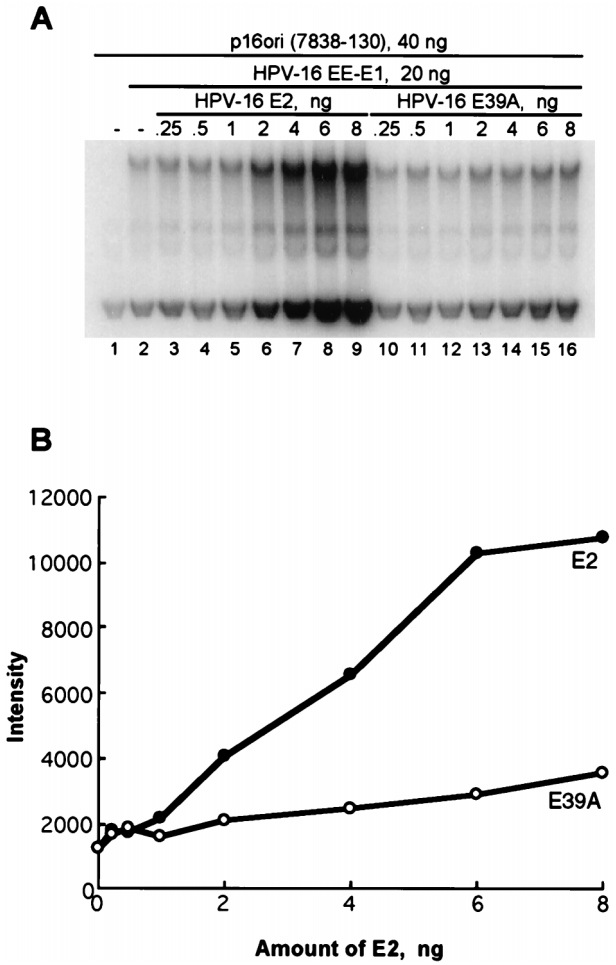

FIG. 2. Interaction of the E2 amino terminus with E1 is required for HPV-16 DNA replication in vitro. (A) Replication of p16ori (40 ng) was determined in the presence of 293 cell extract, 20 ng of EE-E1, and increasing amounts of wild-type HPV-16 E2 (lanes 2 to 9) or E1 interaction-defective E39A HPV-16 E2 (lanes 10 to 16). (B) PhosphorImager quantitation of the replication experiments shown in panel A.

HPV-16 E2 including the E39 residue might encompass a domain sufficient for interaction with E1. Therefore, we reasoned that a peptide encompassing this domain of HPV-16 E2 might inhibit interaction between HPV-16 E1 and E2, thereby inhibiting HPV-16 DNA replication (Fig. 3). To test this possibility, HPV-16 E2 and epitope-tagged full-length HPV-16 E1 (EE16-E1) proteins were expressed separately, using baculovirus vectors. EE-E1 and associated E2 proteins were immunoprecipitated by using an anti-EE epitope monoclonal antibody. Precipitated E2 was detected by Western blotting with polyclonal antiserum raised to the DNA binding domain of HPV-16 E2 (35). As shown in lane 2 of Fig. 4A, E2 coimmunoprecipitated with EE16-E1 but was not precipitated by the EE epitope antibody in the absence of EE16-E1 (lane 1). Peptides encompassing HPV-16 E2 amino acids 29 to 51 (E2N-WP23) or residues 33 to 47 (E2N-WP15) of HPV-16 E2 were then tested for inhibition of E1-E2 interaction. These peptide sequences were derived from the sequence of HPV-16 E2 and were based on an alignment of amino-terminal residues that are conserved among the papillomavirus E2 proteins (Fig. 3). Lanes 3 to 9 of Fig. 4A (top) show the inhibitory effects of increasing concentrations of the E2N-WP23 peptide. The smaller E2N-WP15 peptide displayed comparable inhibition of E1-E2 interaction (Fig. 4A, bottom, lanes 3 to 9). Both the HPV-16 E2 protein itself and the E2-derived peptides act specifically in these assays, since the E39A replication-defective HPV-16 E2 mutant bound only slightly to EE16-E1 (lanes 14). Furthermore, 23-mer and 15-mer peptides containing an amino acid substitution analogous to that of the HPV-16 E2 E39A mutant did not inhibit E1-E2 interaction at concentrations at which the wild-type peptide could do so (lanes 10 to 13). These peptides are named E2N-MP23 and E2N-MP15, respectively (Fig. 3). Quantitation of these results is shown in

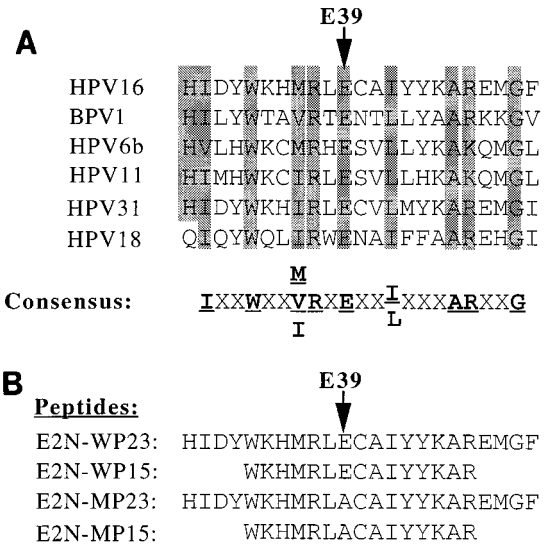

FIG. 3. Derivation of candidate HPV-16 E1-E1 interaction-inhibitory peptides based on conserved residues within the E2 amino terminus. Mutagenesis studies identified residue E39 of HPV-16 E2 as critical for interaction with E1. (A) Alignment of conserved proximal sequences in other papillomavirus E2 proteins. A deduced consensus sequence is shown below in panel A. (B) Sequences of the synthetic peptides derived from this portion of HPV-16 E2. WP peptides retain the conserved glutamic acid at the position analogous to E39 of HPV-16 E2, whereas MP peptides contain a substituted alanine at this position.

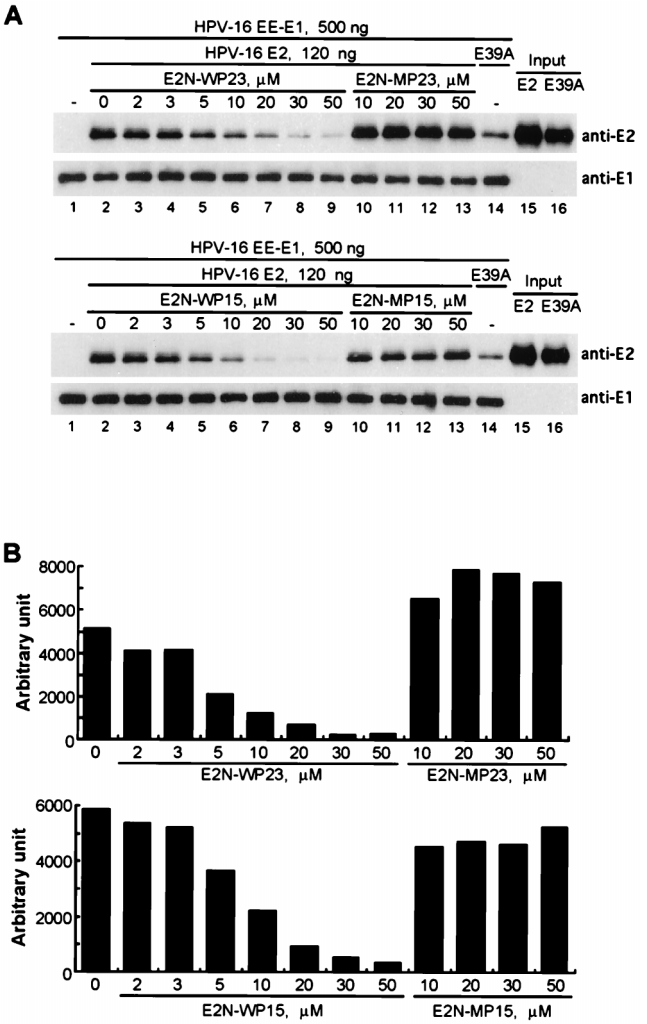

FIG. 4. Peptides derived from the HPV-16 E2 amino terminus inhibit in vitro interaction between HPV-16 E1 and E2. (A) Indicated amounts of epitope-tagged HPV-16 E1 (EE-E1) and HPV-16 E2 were incubated in NET-gel buffer in the presence of increasing concentrations of the 23-mer (E2N-WP23; top, lanes 2 to 9) and the 15-mer (E2N-WP15; bottom, lanes 2 to 9). Binding of EE-E1 to E2 was determined by immunoprecipitation with an anti-EE epitope monoclonal antibody, and E2 was detected by using anti-HPV-16 E2C serum (31). Precipitation of E1 in each reaction was confirmed by probing the same blot with polyclonal antiserum recognizing HPV-16 E1. Increasing amounts of the E2N-MP23 (top) and E2N-MP15 (bottom) peptides were used in lanes 10 to 13. Lane 14 represents the level of interaction observed between the HPV-16 E39A mutant protein and EE-E1. Input wild-type HPV-16 E2 and E39A mutant HPV-16 E2 proteins are shown in lanes 15 and 16. (B) PhosphorImager quantitation of the E2-reactive bands from the experiments shown in panel A.

Fig. 4B. The 50% inhibitory concentrations (IC_{50}) of E2N-WP23 and E2N-WP15 for inhibition of E1-E2 interaction in these assays were 5 and 7.5 μM, respectively. In addition, when inhibition of E1-E2 interaction by a peptide was plotted as a function of the substrate concentration, this analysis indicated that the E2N-WP23 and E2N-WP15 peptides act through competitive inhibition (data not shown). We have previously shown that a domain spanning amino acids 421 to 647 of the HPV-16 E1 carboxy terminus is necessary and sufficient for E2 interaction (45). Specific and comparable inhibition of E1-E2 interaction was also observed in analogous experiments examining the ability of these peptides to inhibit interaction between the HPV-16 E2 protein and the epitope-tagged HPV-16 E1 C terminus (amino acids 421 to 647; data not shown).

Since the E2N-WP23 and E2N-WP15 peptides could inhibit interaction between HPV-6 E1 and E2, their ability to inhibit in vitro HPV-16 DNA replication was also tested (Fig. 5A and B). Figure 5C shows the compiled quantitative results of several independent experiments. Both the 23-mer and 15-mer forms of the wild-type peptide could effectively inhibit in vitro replication. The inhibitory effects of E2N-MP23 and E2N-MP15 were also tested as specificity controls. Each of the wild-type peptides inhibited replication much more effectively than the respective E39A-substituted analog, a result consistent with the importance of the E39 residue of HPV-16 E2 in E1 interaction. The replication-inhibitory effects of the peptides are a result of inhibition of E1-E2 interaction, since the peptides had no effect on the low levels of replication observed with E1 alone (data not shown). Smaller peptides derived from sequences within E2N-WP15 did not inhibit HPV-16 E1-E2 interaction or replication in vitro (data not shown).

Peptide inhibition of in vitro E1-E2 interaction and replication is not limited to HPV-16. Very little is known about the physical properties of domains within the papillomavirus E1 and E2 proteins that are involved in intermolecular recognition and association. Within the sequence of the HPV16-E2-derived 15-amino-acid minimal peptide that inhibits HPV-16 E1-E2 interaction, there are seven well-conserved residues (W-X-X-M/V/I-R-X-E-X-X-I/L-X-X-X-X-A-R) (see also Fig. 3). We reasoned that if these residues adequately represented a conserved E1 interaction domain, the HPV-16 E2-derived peptide might also inhibit interaction between other HPV E1 and E2 proteins. We therefore tested the inhibitory effects of the E2N-WP15 peptide by using the HPV-11 E1 and E2 proteins. Epitope-tagged versions of the HPV-11 (HPV-11 EE-E1) and HPV-16 E1 (HPV-16 EE-E1) proteins were tested for binding to HPV-11 and HPV-16 E2. The effect of the E2N-WP15 peptide on these interactions was also assessed. As shown in Fig. 6A, E2N-WP15 effectively inhibited interaction

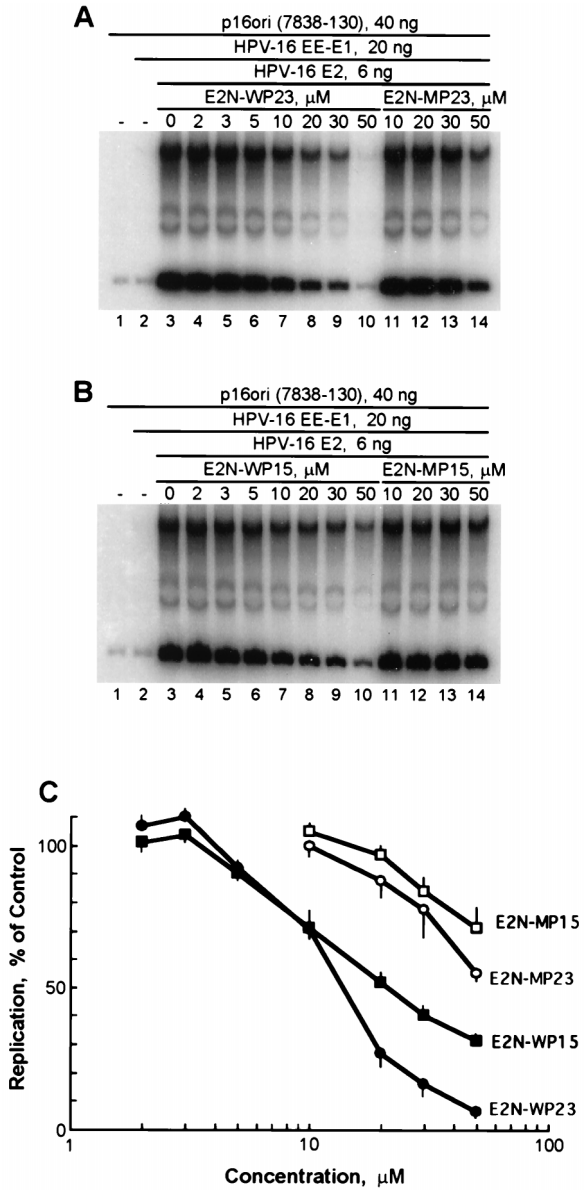

FIG. 5. Peptides derived from the HPV-16 E2 amino terminus inhibit HPV-16 replication in vitro. (A and B) In vitro inhibition of HPV-16 DNA replication was determined by using 40 ng of EE-E1 and 6 ng of HPV-16 E2 in the presence of increasing amounts of E2N-WP23 (A) or E2N-WP15 (B). Negative controls included reaction mixtures with only 293 cell extract and p16ori (lanes 1 in panels A and B), EE-E1 in the absence of E2 (lanes 2 in panels A and B), or complete replication reaction mixtures containing increasing concentrations of E2N-WP23 and E2N-WP15 (panels A and B, lanes 11 to 14). (C) PhosphorImager quantitation of the replication experiments shown in panels A and B. The amount of replication relative to that in lanes 3, in the absence of an inhibitory peptide, is plotted logarithmically as a function of the peptide concentration.

between HPV-11 E1 and HPV-16 E2 (lanes 10 to 13), along with the HPV-11 E1-E2 interaction (lanes 20 to 24), with an IC_{50} of approximately 10 μM. The E2N-WP15 peptide also inhibited interaction between HPV-11 E1 and E2, although this effect was weaker than that observed when other combinations of E1 and E2 were used. (We estimate the IC_{50} of this inhibition to be approximately 20 μM.) As was the case for interaction between HPV-16 E2 and HPV-16 E1, the HPV-16 E2 E39A mutant did not bind HPV-11 E1 (lanes 8 and 16).

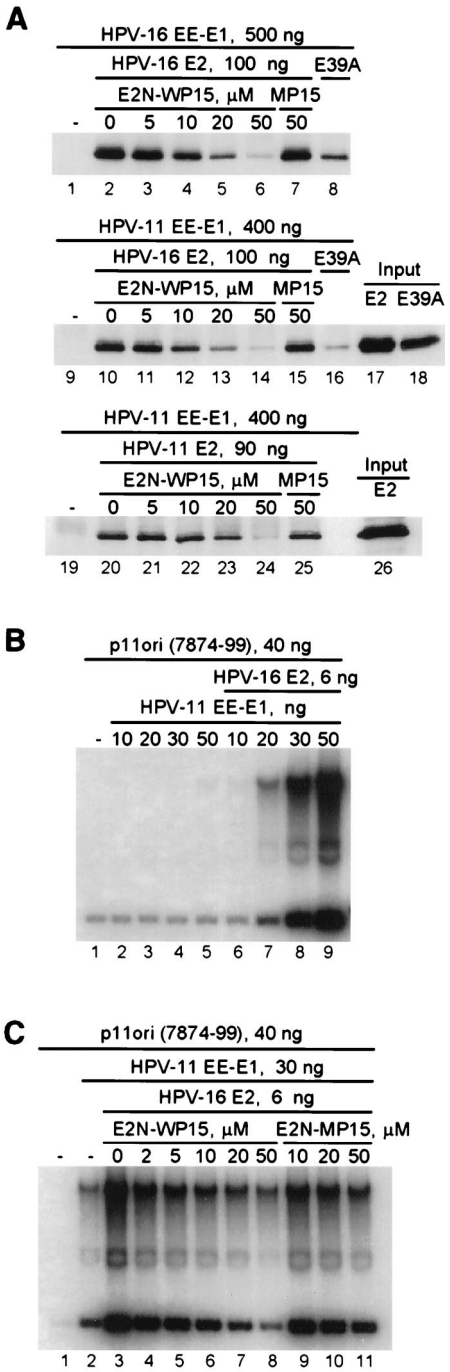

FIG. 6. E2N-WP15 inhibits E1-E2 interaction and in vitro DNA replication of HPV-11. (A) Inhibition of E1-E2 interaction was compared between HPV-16 E1 and HPV-16 E2 (lanes 1 to 8), between HPV-11 E1 and HPV-16 E2 (lanes 9 to 18), and between HPV-11 E1 and HPV-11 E2 (lanes 19 to 26). Epitope-tagged E1 proteins were precipitated by using an anti-EE epitope monoclonal antibody, and associated E2 proteins were detected by Western blotting with anti-E2 serum. Negative control reactions containing EE-E1 with no E2 are shown in lanes 1, 9, and 19. The amounts of input HPV-16 E2 and E39A used in lanes 1 to 16 are shown in lanes 17 and 18. The input amount of E2 used in the HPV-11 E2 experiment (lanes 19 to 25) is shown in lane 26. (B) In vitro replication of HPV-11 origin plasmid p11ori(7874-99) using increasing amounts of purified HPV-11 EE-E1 in the presence (lanes 6 to 9) or absence (lanes 2 to 5) of 6 ng of HPV-16 E2. The reaction mixture in lane 1 contained no HPV proteins. (C) The ability of E2N-WP15 to inhibit in vitro replication of the HPV-11 origin using HPV-11 EE-E1 and HPV-16 E2 was tested (lanes 4 to 8). Effects of equivalent concentrations of the E2N-MP15 peptide were also determined (lanes 9 to 11). Lanes 1 to 3 contained no HPV protein.

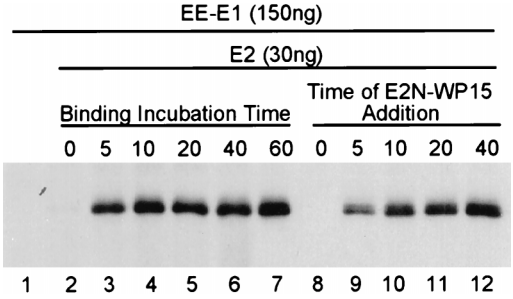

FIG. 7. Kinetics of interaction between HPV-16 EE-E1 and E2 in in vitro binding assays. In vitro E1-E2 binding assays were carried out for the indicated times (lanes 2 to 7), and E1-E2 complexes were determined by immunoprecipitation with an anti-EE monoclonal antibody, followed by Western blotting with antiserum to the C terminus of HPV-16 E2. In parallel reactions, the E2N-WP15 peptide (50 μM final concentration) was added to the E1-E2 binding reaction mixture at the indicated times (lanes 8 to 12), and the reactions were continued through termination at 60 min, when binding between EE-E1 and E2 was determined as described for Fig. 6A.

The E2N-MP15 peptide did not inhibit HPV-11 E1-E2 interaction or interaction between HPV-11 E1 and HPV-16 E2. This suggests that the domain of HPV-16 E2 that interacts with its natural counterpart in HPV-16 E1 can also interact specifically with HPV-11 E1. Interaction between HPV-16 E1 and HPV-11 E2 was not observed (data not shown). This is consistent with the inability of HPV-16 E1 and HPV-11 E2 to support transient replication in cotransfection assays (47). Thus, although many elements of E1-E2 interaction are conserved among the HPV subtypes, these interactions are not completely interchangeable. Further examination of the nature of this nonreciprocal functionality may reveal important determinants in the specificity of E1-E2 interaction.

The ability of the E2N-WP15 peptide to inhibit HPV-11 E1-mediated replication in vitro was also tested. As shown in Fig. 6B (lanes 1 to 9), HPV-11 E1 could cooperate with HPV-16 E2 in supporting dose-responsive in vitro replication of a plasmid containing the HPV-11 origin. (These proteins also functioned in the replication of the HPV-16 origin but less efficiently than in the replication of the HPV-11 origin; data not shown.) The E2N-WP15 peptide inhibited HPV-16 E2–HPV-11 E1-dependent replication of the HPV-11 origin with approximately the same effectiveness as it inhibited HPV-16 E1-E2-mediated in vitro replication (Fig. 5B, lanes 11 to 16). The E39A HPV-16 E2 mutant had only a slight effect on replication in these assays (lanes 17 to 19). Thus, the effects of inhibitory peptides on in vitro replication mirrors those observed in E1-E2 interaction assays.

The dynamics of E1-E2 interaction. Little is known about the ordered assembly of the papillomavirus E1 and E2 proteins at the origin of replication and the formation of the replication initiation complex. Two types of BPV E1-E2-origin complexes have been observed. One complex contains both BPV E1 and E2 (22, 34); the other complex contains only E1, which forms an oligomeric ring around the DNA (33). Although the latter complex can form at high E1 concentrations in the absence of E2 (22, 34), E2 stimulates assembly of the oligomeric E1 complex.

As a preliminary step in examining the assembly of replication factors in HPV-16 DNA replication, we performed experiments to determine the timing of E1-E2 complex formation. In these experiments, EE-E1 was prebound to the EE epitope antibody and protein G beads prior to the indicated additions of E2 protein and an inhibitory peptide. As shown in Fig. 7, HPV-16 E1-E2 complexes form rapidly; the binding reaction was 50% complete after approximately 3 min, and this binding reached a saturated steady state within 10 min (lanes 2 to 7). We also sought to utilize the E2N-WP15 peptide to study the E1-E2 binding reaction itself by adding the peptide (50 μM final concentration) at various times after starting the E1-E2 binding reaction. Addition of the peptide at 5 min resulted in significant inhibition of the subsequent E1-E2 interaction, but addition of the peptide after 10 min had little or no effect on the E1-E2 interaction. We interpret this to mean that complex formation between HPV-16 E1 and E2 is rapid and that E2N-WP15 inhibits the formation of this complex but has no effect upon the E1-E2 complex in the binding reaction after its formation.

DISCUSSION

The papillomavirus E1 and E2 proteins carry out the critical step necessary for viral DNA replication: assembly of the replication initiation complex at the origin of replication. This process requires specific interaction of the E2 amino terminus with the E1 protein. Papillomavirus replication is otherwise dependent on host cell polymerases and other components of the host cell replication machinery. Little is known about the formation and composition of this replication initiation complex or about the subsequent onset of elongation and termination. A greater understanding of the regulation and coordination of papillomavirus DNA replication would enhance our knowledge of both basic mechanisms of DNA replication in mammalian cells and the specific manner in which papillomavirus DNA replication is carried out.

We have mapped a number of viral DNA replication-associated activities within the HPV-16 E1 protein (45). The E2 interaction domain of HPV-16 E1 resides within the carboxy-terminal 223 amino acids (amino acids 421 to 647), a region that also includes the ATP-binding domain. This region is also capable of interacting with other E1 molecules in two-hybrid assays (46). Many missense mutations within this domain of HPV-16 E1 simultaneously disrupt multiple E1 activities, including transient replication, E1-E1 association, formation of E1-E2 complexes, ATPase activity, and the ability of E1 to interact with cellular proteins in two-hybrid assays. The pleiotropic effects of many of these HPV-16 E1 mutations have led us to speculate that such E1 functions are tightly clustered or functionally interdependent (46).

The initial deletion mapping studies of the BPV type 1 (BPV-1) E2 amino terminus identified domains necessary for transcriptional activation and viral DNA replication (24, 42) but failed to discriminate between these two E2 functions. Nonoverlapping deletions within the conserved amino terminus of BPV E2 (e.g., Δ1-15, Δ92-161, and Δ195-282) disrupted both transcriptional activation and replication functions. These results implied either that the E2 transcriptional activation and replication functions coexist within an extended domain of the amino terminus or that such deletion mutations cause concurrent disruption of multiple functional moieties within the amino terminus.

More recent studies of the HPV-16 E2 and BPV-1 E2 amino-terminal domains that employed alanine substitution mutagenesis successfully separated the E2 amino-terminal replication and transcriptional activation functions. Mutation of the conserved glutamic acid residue at position 39 of HPV-16 E2 to alanine specifically abrogates its ability to stimulate transient papillomavirus replication. This mutant no longer interacts with E1 but is proficient in transcriptional activation. Alternatively, substitution of alanine for the conserved isoleucine at position 73 of HPV-16 E2 abrogates transcriptional activa-

tion but has no effect on E1 interaction and DNA replication (31). Similar directed mutagenesis studies have extended these results to BPV E2 (1, 6, 7, 10). A genetic screen based on BPV E2 transcriptional activation in yeast has identified additional E2 mutants that have lost either or both of these functions (6, 13).

Such studies have provided convincing evidence that the papillomavirus E2 amino terminus is composed of at least two separable functional domains: one domain, defined by isoleucine 73, that is required for transcriptional activation and a separate domain, defined by the glutamic acid residue at position 39, that is required for E1 interaction and replication. In this sense, it is notable that the BPV E2 deletion mutants mentioned above that disrupted both transcriptional activation and replication overlap neither the E39 nor the I73 domain. Indeed, even missense mutations scattered throughout the first 200 amino acids of E2 are capable of abrogating either or both functions (1, 6, 13, 31, 42). Thus, although the transcriptional activation and replication functions of the E2 amino terminus are functionally independent, the domains required for these respective functions do not necessarily retain their functional integrity when removed from their normal context. Furthermore, in our mapping studies of HPV-16 E2, we defined a domain encompassing amino acids 1 to 190 as the smallest domain capable of interaction with HPV-16 E1 in two-hybrid assays (45). For these reasons, it was uncertain whether a peptide derived from the E39 region of HPV-16 E2 would be capable of competing with intact E2 proteins for specific interaction with the HPV-16 E1 carboxy terminus. In this study, we have demonstrated that a 15-amino-acid peptide derived from the amino terminus of HPV-16 E2 and centered upon E39 can inhibit both HPV-16 E1-E2 interaction and papillomavirus replication in vitro. This inhibition is specific, since an equivalent peptide containing an alanine substitution at position 39 had minimal inhibitory activity in both assays. The ability of this peptide to act as a competitive inhibitor of in vitro E1-E2 interaction suggests that a specific E1 recognition activity is contained within a relatively small part of the E2 amino terminus and that this domain is able to adopt a conformation suitable for occupancy of the E2 binding site of E1.

The E1-E2 interaction and peptide inhibition experiments presented here indicate functional conservation in E1-E2 recognition and interaction between the E1 and E2 proteins of HPV-11 and HPV-16. The E39 residue of E2 appears to be a conserved functional component of E1-E2 interaction among these proteins, since this residue is required for interaction between HPV-16 E1 and E2, as well as HPV-11 E1 and HPV-16 E2. E39 is also critical for the replication function of BPV-1 E2 (10). Our peptide inhibition experiments have reiterated the importance of this residue in both HPV-16 and HPV-11 E1-E2 interaction and replication, since E2N-WP15 disrupted interaction between all combinations of the HPV-16 and HPV-11 E1 and E2 proteins. The lack of interaction we observed between HPV-16 E1 and HPV-11 E2 in binding assays is consistent with the inability of these proteins to support transient replication when transfected into 293 cells (47). HPV-16 E1 seems to be particularly selective in selection of its E2 partner, compared to HPV-11 E1, which can cooperate functionally in replication assays with the E2 proteins of HPV-6, HPV-11, HPV-16, or HPV-18 (9, 38). Experiments using HPV-11 and HPV-16 chimeric E1 proteins have identified the carboxyl-terminal 284 amino acids of HPV-16 E1 as the domain that mediates selective binding of HPV-16 E1 to HPV-16 E2 (47). This selectivity has been attributed to steric interference within this domain of HPV-16 E1 that somehow prevents HPV-11 E2 binding. Testing the ability of an extended set of peptides derived from the E39 region of a variety of E2 proteins to inhibit interaction between other E1 and E2 proteins may provide greater insight into the exact nature of this selectivity and thus allow an estimation of the spectrum of HPV subtypes against which an individual peptide or small molecule might be effective.

Demonstration that a peptide could inhibit in vitro E1-E2 interaction and papillomavirus DNA replication constitutes an essential first step in assessing the potential efficacy of small compounds as papillomavirus antiviral agents and validates this approach to the design of papillomavirus antiviral compounds. We also used the E2N-WP15 peptide to examine early events in the formation of E1-E2 complexes and initiation of viral DNA replication. Formation of the HPV-16 E1-E2 complex occurred quickly, within 10 min, under the conditions of both of the in vitro E1-E2 interaction assays. We have not tested the ability of these peptides to inhibit HPV DNA replication in cell-based assays; peptides composed of conventional amino acids are not themselves typically useful as pharmacological reagents, since they are often unstable or unable to enter cells. We will explore the incorporation of peptide modifications known to increase peptide solubility, permeability, and bioavailability (e.g., rhodamine conjugation or amide bond methylation) to facilitate the use of the E2N-WP15 peptide or similar peptides to inhibit HPV DNA replication in vivo. Regardless of whether E2N-WP15 or similar inhibitory peptides are effective replication inhibitors in vivo, the peptides defined in these studies may be useful in defining structures that would serve as a starting point for the design or discovery of clinically useful antiviral compounds that inhibit papillomavirus replication.

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